

Mechanisms of Multistep Carcinogenesis and Carcinogen Risk Assessment

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Many different types of chemical exposures can increase the incidence of tumors in animals and humans, but usually a long period of time is required before the carcinogenic risk of an exposure is manifested. Both of these observations can be explained by a multistep/multigene model of carcinogenesis. In this model, a normal cell evolves into a cancer cell as the result of heritable changes in multiple, independent genes. The two-stage model of initiation and promotion for chemical carcinogenesis has provided a paradigm by which chemicals can act by qualitatively different mechanisms, but the process of carcinogenesis is now recognized as more complex than simply initiation and promotion. Even a three-stage model of initiation, promotion, and progression, which can be operationally defined, is not adequate to describe the carcinogenic process. The number of genes altered in a cancer cell compared to a normal cell is not known; recent evidence suggests that 3–10 genetic events are involved in common adult malignancies in humans. Two distinct classes of genes, proto-oncogenes and tumor-suppressor genes, are involved in the cancer process. Multiple oncogenes may be activated in a tumor, while multiple tumor-suppressor genes may be inactivated. Identification of the genes involved in carcinogenesis and elucidation of the mechanisms of their activation or inactivation allows a better understanding of how chemical carcinogens influence the process of neoplastic evolution. The findings of multiple genetic changes (including point mutations, chromosomal translocations, deletions, gene amplification, and numerical chromosome changes) in activated proto-oncogenes and inactivated tumor-suppressor genes provide experimental support for Boveri's somatic mutation theory of carcinogenesis. In addition to mutagenic mechanisms, chemicals may heritably alter cells by epigenetic mechanisms and enhance the clonal expansion of altered cells. Most chemical carcinogens operate via a combination of mechanisms, and even their primary mechanism of action may vary depending on the target tissues. The classification of chemicals by mechanism of action or by nongenotoxic or genotoxic activity has certain inherent difficulties because no classification of chemicals is exhaustive or definitive.

Carcinogenesis Is a Multistep Process

Cancer remains a major chronic health problem associated with toxicological substances. The long latency period of cancer induction (years in rodents and decades in humans) is a major problem in the evaluation of toxicological hazards and risk assessment. We understand, at least in part, the underlying reasons for the time requirement of cancer formation. It is now clear that for a normal cell to evolve into a cancer cell, multiple heritable changes within the cell are required, i.e., carcinogenesis is a multistep process involving multiple genes. Several lines of evidence support the conclusion that chemical carcinogenesis is a multistep process. These are listed in Table 1 and discussed in detail elsewhere (1).

One of the underlying premises of most multistep models of carcinogenesis is that genetic and/or epigenetic alterations of multiple, independent genes are involved. Although the process of chemical carcinogenesis is often separated operationally into three stages, i.e., initiation, promotion, and progression (2), the number of genetic changes involved in each of these operationally defined stages has not yet been determined.

Initiation involves the induction of an irreversibly altered cell and is frequently equated with a mutational event. This con-

Table 1. Evidence for multistage models of carcinogenesis.*

Histopathological observations of tumors reveal multiple stages of tumor progression such as dysplasia and carcinoma <i>in situ</i> .
Two-stage model of chemical carcinogenesis in mouse skin shows that different chemicals affect qualitatively different stages in the carcinogenic process.
Individuals with genetic traits manifested by an early occurrence of cancer (e.g., familial retinoblastomas, adenomatosis of the colon and rectum) suggest that one step in the carcinogenic process can be a germline mutation, but additional somatic events are required for neoplastic development.
Mathematical models based on age-specific tumor incidence curves are consistent with 3–7 independent hits required for tumors.
Cell culture studies with chemical carcinogens reveal that different phenotypic properties of a tumor cell are acquired by a progressive process.
Cell culture studies with viral and tumor-derived oncogenes show that neoplastic conversion of normal cells generally requires multiple cooperating oncogenes. In contrast, certain preneoplastic (immortal) cells are neoplastically transformed by a single oncogene.
Transgenic mice that carry activated protooncogenes in their germline develop focal tumors, which are apparently monoclonal in origin, suggesting that additional somatic events are required for full malignant progression.

*See Barrett (12) for a more complete discussion and references.

clusion is supported by the findings of mutational activation of *ras* proto-oncogenes in rat mammary carcinomas, mouse skin papillomas, and mouse hepatomas (3–7). The mechanisms of initiation may vary, however, in different tissues or with different initiators in the same tissue (8). Promotion is the experimentally defined process by which the initiated cell clonally expands into

a visible tumor, often a benign lesion such as a papilloma. This process undoubtedly involves at least some epigenetic factors that selectively influence the proliferation of the initiated cell. Whether genetic mechanisms are also involved in tumor promotion is unclear. The end products of tumor promotion are generally benign lesions or foci of preneoplastic cells. These cells must undergo one or more additional heritable changes during the progression to a malignant neoplasm. The progression of benign tumors to malignant cancers is a phase in carcinogenesis clearly distinct from promotion (9,10).

In the past decade, there have been tremendous advances in our understanding of the target genes in carcinogenesis (11,12). Two classes of genes, proto-oncogenes and tumor-suppressor genes, are involved in the evolution of most, if not all, cancers (Table 2). Proto-oncogenes, when activated by mutational mechanisms, result in positive proliferative signals for tumors. Tumor-suppressor genes, in contrast, block the neoplastic growth of cells by undefined mechanisms and therefore must be inactivated or lost in tumor cells (13). In most common human tumors (e.g., lung, colon, and breast) multiple tumor-suppressor genes are frequently affected, indicating that malignant growth is subject to several levels of negative control (12,14,15).

The number of genes involved in neoplastic development is not known with certainty. Most colorectal cancers have three or more altered genes (16,17), and estimates of as many as 10 or more mutational changes have been proposed to occur in adult human cancers (13). These findings are consistent with multistep models developed on the basis of specific incidence rates of cancers increasing exponentially with the fifth to seventh power of age (18). Analysis of multistep carcinogenesis at the molecular level, therefore, indicates that the process of neoplastic evolution is significantly more complicated than the relatively simple two-stage (initiation and promotion) model of carcinogenesis or

even a three-stage model of initiation, promotion, and progression. As an example, the model described by Vogelstein and co-workers for colorectal cancers (Fig. 1) shows that multiple genetic changes must occur after the promotion or clonal growth of the initiated cells (16,17). Thus, the progression phase of carcinogenesis represents multiple stages at which chemicals might influence the neoplastic process (19).

There are three general mechanisms by which a substance can influence the multistep, carcinogenic process (Table 3). A substance can induce a heritable alteration in one or more critical genes in the multistep process by one of two mechanisms. This heritable change may have either a genetic or epigenetic basis. Although considerable insight into the mechanisms of genetic changes by chemicals exists, little is known about the mechanisms of carcinogen-induced epigenetic, heritable changes. A third mechanism by which a substance can influence multistep carcinogenesis is the facilitation of clonal expansion of an initiated or intermediate cell, which increases the probability of additional, spontaneous (mutational or epigenetic) heritable changes.

Mutagenesis as a Mechanism of Carcinogenesis

The origin of the somatic mutation theory of carcinogenesis is generally credited to Theodor Boveri, who in 1914 published his book (20) *Zur Frage der Entstehung Maligner Tumoren* [On the Problem of the Origin of Malignant Tumors]. The English translation of this book by his wife, Marcella Boveri, was published in 1929. Boveri's hypothesis on the origin of malignant tumors was extraordinarily comprehensive and included many predictions, which subsequently have been proven true. For these reasons, Boveri is generally acknowledged as the father of the somatic mutation theory of carcinogenesis. There is now considerable evidence to support this theory, as discussed elsewhere (21).

Genetic changes can be classified as either gene mutations, chromosome rearrangements, gene amplification, or aneuploidy. There are now clear examples of each of these mutational changes in different tumors (Table 4), which provide critical support for the somatic mutation theory of carcinogenesis. Point mutations have been observed to activate, proto-oncogenes and to inactivate tumor-suppressor genes in certain cancers. Chromosome rearrangements of oncogenes are also well documented. Gene amplification as well as numerical chromosome changes are important in a number of different cancers (21). Therefore, chemicals that induce any one of these four distinct types of genetic events could heritably alter a critical target gene necessary

Table 2. Two classes of genes involved in carcinogenesis.

Proto-oncogenes	Tumor-suppressor genes
Involved in cellular growth and differentiation	Function unknown but possibly involved in cellular growth and differentiation (negative regulators of cell growth?)
Family of genes exists	Family of genes exists
Activated (quantitatively or qualitatively) in cancers	Inactivated or lost in cancers
Activation by point mutation, chromosome translocation, or gene amplification	Inactivation by chromosome loss, chromosome deletion, point mutation, somatic recombination of gene conversion
Little evidence for involvement in hereditary cancers	Clear evidence for involvement in hereditary and nonhereditary cancers

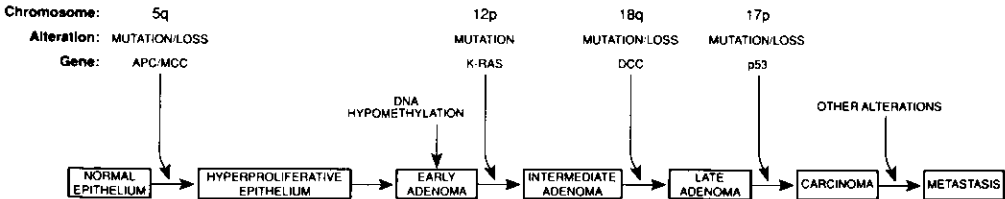


FIGURE 1. A model for colorectal cancer based on the work of Vogelstein and colleagues (16).

Table 3. Mechanisms by which a substance can influence multistep carcinogenesis.

By inducing heritable mutation in a critical gene
By inducing heritable, epigenetic change in a critical gene
By increasing clonal expansion of a cell with a heritable alteration in a critical gene, allowing for increased probability of additional events

Table 4. Examples of molecular, genetic, and cytogenetic changes in tumors.

Type of genetic change	Examples
Gene mutation	Point mutation (G→T) in codon 12 of the c-Ha-ras gene in EJ/T24 bladder carcinoma Point mutation (A→G) in the splice acceptor sequence of exon 21 in the retinoblastoma gene of J82 bladder carcinoma cells
Chromosome rearrangement	Philadelphia translocation (t(9;22) in chronic myelogenous leukemia, t(8;14) in Burkitt's lymphoma
Gene amplification	N-myc gene in neuroblastomas, c-myc gene in lung carcinomas, neu gene in mammary carcinomas
Aneuploidy	+12 in chronic lymphocytic leukemia, +8 in acute nonlymphocytic leukemia, blast phase of chronic myelogenous leukemia, +15 in murine T-cell leukemias, -22 in meningiomas, -15 in Syrian hamster tumors induced by transfection of v-Ha-ras and v-myc

for neoplastic development. These observations support the use of mutagenicity assays in the evaluation of carcinogenic risks of chemicals to humans.

The mechanisms of carcinogen-induced activation of oncogenes have been elucidated, and the implications of these findings are highly important for risk assessment of chemicals, which can be illustrated by three examples: *a*) Carcinogen-induced point mutations, resulting in activation of a *ras* oncogene, have been demonstrated in carcinogenesis of skin (4), mammary gland (5), and liver (6,7). In these model systems, the data support the conclusion that these point mutations are the critical changes in the initiation of these tumors. These findings provide experimental evidence for using the linear dose-response curves observed in mutagenesis studies for carcinogen risk assessment in the absence of pharmacokinetic and other data. *b*) Elucidation of oncogene activation by other genetic changes (Table 4) such as chromosome rearrangements and gene amplification provides a theoretical framework for the use of these end points in risk assessment. *c*) The observations that normal cells are not neoplastically transformed by a single oncogene but rather require two or more cooperating oncogenes and inactivation of multiple tumor-suppressor genes support a multistep or multihit model of carcinogenesis (22–24) and have significant implications for risk assessment of chemicals. Because at least multiple mutations must occur for a tumor cell to arise and these mutations may occur by different genetic mechanisms, it is not surprising that a single toxicological end point, such as carcinogen-DNA adducts, does not always correlate with carcinogenic potency of chemicals.

Tumor Promotion and Tumor Progression

The multistep/multigene model of carcinogenesis provides insights into many important features of cancer development and carcinogen risk assessment. The necessity for a malignant cell to acquire multiple, heritable alterations at independent genetic loci explains, at least in part, the long latency period for cancer. This model also explains how noncarcinogenic substances can influence the carcinogenic process. Chemicals that influence the clonal proliferation of initiated or other intermediate cells in the neoplastic process may increase the risk of cancer development in exposed populations. Conversely, chemicals that are highly mutagenic but do not induce cell proliferation may be noncarcinogenic. The carcinogenicity of these chemicals will depend, however, on the state of proliferation of the target tissue. For example, polycyclic hydrocarbons, aromatic amines, and nitrosoamines are highly carcinogenic in the livers of neonatal mice, but the same exposures to adult mice are noncarcinogenic in the liver due to a lack of cell proliferation (25).

The end product of tumor promotion is generally a benign lesion or foci of preneoplastic cells. These cells must undergo one or more additional heritable changes during the progression to a malignant neoplasm. The progression of benign tumors to malignant cancers is a phase in carcinogenesis clearly distinct from promotion. This conclusion is supported by a number of observations. Malignant tumors are distinct from benign tumors or other preneoplastic lesions in terms of their histopathological characteristics of cellular morphology, invasiveness, growth, and differentiation. The stages of promotion and progression can also be distinguished on the basis of differential responses to certain chemical treatments. In initiation-promotion experiments on mouse skin, the incidence of carcinomas is not necessarily proportional to the number of papillomas (26–32). Telocidin, an indole alkaloid, induces more carcinomas, but fewer papillomas, than the phorbol ester promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mouse skin (28). Mezerein is a weak promoter of epidermal papillomas in SENCAR mice, but it yields a similar number of carcinomas as the potent promoter TPA (31). Likewise, the free-radical-generating chemical benzoyl peroxide is only moderately active as a promoter of papillomas but is far more active than TPA in inducing malignant tumors (32). Finally, the anti-inflammatory steroid fluocinolone acetonide inhibits papillomas initiated by DMBA and promoted by 7-bromomethylbenz[*a*]anthracene without affecting the carcinoma incidence in treated mice (30). These studies clearly indicate that the induction of carcinomas is only in part determined by the number of the benign papillomas.

Tumor promoters, although effective in producing multiple benign tumors or preneoplastic foci, are not particularly effective in influencing the progression of these lesions to malignant neoplasms in many experimental models (10,34). For example, in the mouse skin model, phorbol ester tumor promoters influence malignant progression by increasing the number of precursor lesions (i.e., papillomas), but do not directly induce the transition of papillomas to carcinomas (10,32). Treatment of benign tumors with alkylating and other mutagenic agents increases the frequency and rate of malignant conversion (19,32). The promoter TPA is ineffective in enhancing malignant pro-

gression, but other promoters (e.g., benzoyl peroxide and telocidin) may effect both promotion and progression (28,33). These observations caution against using premalignant lesions alone for carcinogen risk assessment of tumor promoters because in certain cases the incidence of benign tumor may greatly overestimate or underestimate the risk for malignant cancers.

The evolution of malignant tumors from benign lesions involves the acquisition of one or more qualitative changes in the precursor cells. In fact, progression probably involves multiple, heritable changes. In mouse skin, papillomas display no histopathological evidence of dysplasia after 10 weeks of tumor promotion with phorbol esters (35,36); however, at later times (20–40 weeks of promotion), the papillomas show evidence of moderate to severe dysplasia and, concomitantly, aneuploid tumor cells are detectable. These phenotypic changes are also observed in the carcinomas that arise from these papillomas (35). In chemically induced rat hepatocarcinogenesis, multiple events are postulated to be involved in the progression phase (37,38). In other tissues, morphological evidence for multiple steps in the progression of dysplastic lesions to carcinomas *in situ* and to malignant carcinomas is well established (39).

From epidemiological studies, some human carcinogens have been shown to affect predominantly late stages in the carcinogenic process (18). This does not necessarily imply that such chemicals operate in a manner similar to tumor promoters in two-stage experimental models. A given chemical may affect events in the progression phase of carcinogenesis, which, as described above, are not affected by classical promoters such as the phorbol esters (19).

Arsenic is an example of a chemical that may act primarily as a tumor promotor, i.e., a chemical that affects the progression stage of carcinogenesis. Arsenic is a well-established carcinogen in humans (40), but there is little evidence for its carcinogenicity in animals (41–43). It is inactive as an initiator or tumor promoter in a two-stage model of epidermal carcinogenesis in mice (44,45). Brown and Chu (46) have proposed that arsenic exposure affects a late stage in the carcinogenic process based on exposure effects in humans. These authors have further postulated that the human data are inconsistent with the hypothesis that arsenic acts during the promotion phase of the carcinogenic process. One of the purported hallmarks of tumor promotion is reversibility (47). However, epidemiological studies of human cancers caused by arsenic exposure fail to show reversibility of the excess lung cancer mortality after exposure ceases (46). Based on these observations, we have proposed that arsenic acts specifically in the progression phase of carcinogenesis (48). This hypothesis is supported by our observation that arsenic is an effective inducer of gene amplification (48) and would explain why arsenic is ineffective as a complete carcinogen, initiator, or tumor promoter. Oncogene amplification has been shown in some tumors to correlate with the degree of neoplastic progression (49–52) and arsenic-induced oncogene amplification may explain the observed increase of tumors at a late stage in human carcinogenesis. These findings emphasize the importance of considering all the steps in the multistep process of carcinogenesis. Carcinogen evaluation based only on the principles of initiation and promotion may not accurately predict the hazards of human carcinogens.

Mechanisms of Known Human Carcinogens

The genetic toxicology of known human carcinogens identified by the International Agency for Research on Cancer (IARC) has recently been reviewed (53–55). The conclusion of these reviews is that most, but not all, human carcinogens are

Table 5. Mutagenicity of International Agency for Research on Cancer group 1 human carcinogens in bacteria and rodent bone marrow tests.^a

Carcinogens	Salmonella mutagenicity	Rodent bone-marrow cytogenetic effects
Organic compounds		
Aflatoxins	+	+
4-Aminobiphenyl	+	+
Analgesics containing phenacetin	+	+
Azathioprine	+	+
Benzene	–	+
Benzidine	+	+
Betel quid and tobacco	+	+
bis(Chloromethyl)ether and chloromethyl ether	+	I
Chlorambucil	+	+
Chlornaphazine	+	+
Cyclophosphamide	+	+
Melphalan	+	+
Methyl-CCNU	+	+
MOPP (and other combined therapies)	+	+
Mustard gas	+	+
Myleran	+	+
2-Naphthylamine	+	+
Tobacco, smokeless	+	+
Tobacco smoke	+	ND
Tresulphan	+	+
Vinyl chloride	+	+
Soots, tars, and oils		
Coal-tar pitches	+	ND
Coal tars	+	ND
Mineral oils, untreated and mildly treated	+	ND
Shale oils	?	?
Soots	+	ND
Hormones^{b,c}		
Diethylstilbestrol	–	+
Estrogen replacement therapy	ND	ND
Estrogens, nonsteroidal	ND	ND
Estrogens, steroidal	ND	ND
Oral contraceptives, combined	ND	ND
Oral contraceptives, sequential	ND	ND
Metals^b		
Arsenic compounds	–	+
Chromium compounds (hexavalent)	+	+
Nickel and nickel compounds	–	ND
Fibers		
Asbestos	–	ND
Erionite	ND	ND
Talc-containing asbestiform fibers	–	–
Other		
8-Methoxypsoralen + UV	+	ND

Abbreviations: CCNU, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; MOPP, nitrogen mustard vincristine procarbazine and prednisone.

^aI, conclusive; ND, no data; +, positive response; –, negative response; ?, responses differ among different members of this group (54).

^bThe determination of carcinogenicity applies to the groups of chemicals as a whole and not necessarily to all chemicals within each group.

^cLimited test results are available, but the group of agents that includes estrogens, progestins, and their combinations typically give negative results in genetic toxicity studies (54).

active in a variety of genetic toxicology tests. Table 5 is a summary taken from Shelby and Zeiger (54) of the mutagenicity of known human carcinogens subdivided into different chemical groups. The first group is the organic compounds, the vast majority of which are active in inducing *Salmonella* mutagenesis and chromosome aberrations or micronuclei in the rodent bone marrow tests *in vivo*. For chemicals not adequately tested, their predicted activity was based on structural alerts (56).

The soots, tars, and oils are also generally active in the *Salmonella* assay, and when tested they are active in other systems, although in many cases these studies have not been done. Some of the carcinogenic metals are not active in the *Salmonella* assay but show activity in a variety of other test systems, in particular, clastogenicity assays. This finding emphasizes the necessity of not limiting testing of mutagenicity to gene mutation assays but rather of examining the full spectrum of possible mutational events.

Two classes of chemicals that are generally inactive in assays for mutagenicity are the mineral fibers and the hormones. Therefore, it is worthwhile to discuss in more detail the mechanisms of action of these two classes of known human carcinogens.

Asbestos Carcinogenicity

Asbestos is clearly carcinogenic in a wide variety of animal and human studies (57). Asbestos and other mineral fibers are generally described as nongenotoxic carcinogens because they are inactive in a variety of short-term tests, particularly gene mutation tests (58). It has been proposed that asbestos must operate as a tumor promoter, not as an initiator, and there are several lines of evidence that are consistent with this hypothesis (58). There is a synergistic interaction between asbestos exposure and smoking for the risk of lung cancer in humans (59). It has been suggested in these cases that smoking is the initiator and asbestos is a promoter. This finding could be explained just as well by assuming that asbestos is the initiator and smoking is the promoter or that smoking is the initiator with some unknown promotional stimulus and that asbestos acts in the later phases of progression by nonpromotional mechanisms. There is also a cocarcinogenic effect of asbestos and various polyaromatic hydrocarbons, and this might be involved in the synergism between smoking and asbestos. Topping and Nettesheim (60) demonstrated that asbestos enhances DMBA-induced carcinomas in the rat tracheal transplant model. In this model, asbestos alone induced a low incidence of tumors; however, there was a clear enhancement of DMBA-induced cancers by asbestos. Whether this is strictly a promotional effect of asbestos remains to be elucidated. Finally, there is a number of observations indicating that asbestos induces cellular and biochemical changes, such as hyperplasia, metaplasia, DNA synthesis, and stimulation of oxygen free-radicals that are typical of known tumor promoters (61,62). By analogy to other promoters, therefore, it has been suggested that asbestos is operating through promotional mechanisms.

On the other hand, there is also evidence that asbestos can operate as an initiating agent. Asbestos is a complete carcinogen in a wide variety of animal models by multiple routes of administration (57,63-65). Epidemiological data in humans suggest that asbestos acts at an early stage in mesothelioma formation,

which would be consistent with an initiation effect (65). Two additional lines of evidence suggest conceptually that asbestos might have initiating potential. Although asbestos is inactive as a gene mutagen, it induces chromosomal mutations, both aneuploidy and aberrations, in a wide variety of mammalian cells including mesothelial cells in culture (66). Asbestos treatment of either human or rodent cells also induces cell transformation, leading to neoplastic progression (58,66).

Asbestos induces morphological and neoplastic transformation of Syrian hamster embryo cells (67). Fiber-induced transformation in this *in vitro* model parallels *in vivo* induction of mesotheliomas in rats in that cell transformation is dependent on fiber dimension. Cell transformation is more effectively induced by long, thin fibers than by short, thick fibers. Reducing fiber length from approximately 15 μm to 2 μm has a dramatic effect on reducing activity, and fibers shorter than 1 μm are essentially inactive (67).

How can asbestos lead to heritable, neoplastic alterations of exposed cells? The mechanism proposed (58,66) is that the asbestos fibers are phagocytized by the cells and accumulate inside the cells around the perinuclear region. When these cells attempt to undergo division, there is physical interference by the fibers with the normal process of mitotic chromosome segregation, leading to anaphase abnormalities, such as chromosome losses, gains, and aberrations (68). A good correlation between the abilities of different fibers to induce cell transformation and chromosomal changes has been reported (68). These changes are random, but the transformed cells have nonrandom chromosomal changes associated with the early stages of transformation. For example, trisomy of chromosome 11 is observed in the majority of hamster cells transformed by asbestos (70). Immortalization (escape from cellular senescence) is an early event in asbestos-induced transformation which involves loss of a normal gene required for senescence. By reintroducing human chromosomes into these transformed cells, the normal process of cellular senescence is restored. The gene involved in senescence has been mapped to a region on the long arm of human chromosome 1 (71). Human mesotheliomas are highly aneuploid and show a wide variety of chromosome changes, but several nonrandom structural and numerical changes have been identified, including changes on chromosome 1 (62). Because there is clear evidence for nonrandom chromosomal changes in mesotheliomas and because asbestos fibers in culture can induce chromosomal alterations, it is reasonable to assume that this mechanism plays some role in the genesis of asbestos-related cancers. This is not likely the sole mechanism of action for asbestos. In fact, the promotional mechanisms mentioned earlier are probably quite important as well. The target cells for mesotheliomas are generally nonproliferative. In order for chromosomal changes induced by asbestos to occur, normal mesothelial cells need to be stimulated to proliferate (72). Both the induction of cell proliferation as well as the subsequent chromosomal changes are probably involved in the asbestos carcinogenicity.

Hormonal Carcinogenicity

Hormones represent another class of important human carcinogens. As reviewed recently by Preston-Martin et al. (73), estrogenic hormones play a major role in the relative risk for different cancers in women, including endometrium, breast, and

ovarian cancer. The natural estrogen 17 β -estradiol increases the incidences of mammary, pituitary, uterine, cervical, vaginal, and lymphoid tumors, and interstitial-cell tumors of the testes in mice; it also increases the incidences of mammary and pituitary tumors in rats and renal tumors in hamsters (74). Perhaps the best-studied human carcinogen in terms of mechanism of action is the synthetic hormone diethylstilbestrol (DES). When DES was given therapeutically to women during pregnancy, their offspring had a higher incidence of clear-cell adenocarcinoma (75). This chemical also induces cancer in a wide variety of animal models. Diethylstilbestrol increases the incidences of mammary tumors, lymphoid tumors, interstitial-cell tumors of the testes, cervical tumors, and vaginal tumors in mice; pituitary, mammary, and bladder tumors in rats; and renal tumors in hamsters (74). Studies on the mechanism of action of DES in different models lead to the conclusion that not one single mechanism but rather multiple mechanisms are involved in the action of this human carcinogen.

Several possible mechanisms by which hormones may influence cancer development are given in Table 6. It has been proposed that estrogens are carcinogenic due primarily to their ability to stimulate cell proliferation. The hormonal dependence of transplantable tumors is consistent with this proposed mechanism of action. This hypothesis is also supported by experimental observations of tumor-promoting effects of estrogens on carcinogen-initiated mammary cancers, liver cancers, and vaginal tumors (74,76). Analyses of the influence of hormonal factors on human breast cancers also indicate an effect on a late stage in the carcinogenic process, consistent with a promotional effect (78). Therefore, there is evidence from several systems in support of the hypothesis that estrogens are epigenetic carcinogens acting via a promoting effect related to stimulation of proliferation of estrogen-responsive cells. In addition, DES heritably reprograms developmental processes and results in marked changes in the expression of the differentiation phenotype of cells in animals following exposure to DES during critical developmental periods (77,78). The mechanisms by which DES induces such striking changes in the entire endometrium are unknown.

Despite the convincing evidence that estrogens have an epigenetic effect on carcinogenesis, there are observations that indicate that estrogens can also induce heritable alterations important in neoplastic development. Diethylstilbestrol induces tumors in humans and experimental animals following single or short-term prenatal exposure (74,79). The offspring of treated animals have increased tumor incidences, even though they are not exposed to further treatment. Newbold et al. (79) have shown that DES treatment of neonatal mice from days 1 to 5 after birth, a time period that corresponds to late prenatal human development, results in a high incidence (90%) of uterine adenocarcinoma at 18 months of age. In this model, tumors are induced by brief treatments or even a single injection of DES.

There is also evidence that estrogenic activity is not sufficient to explain the carcinogenic activity *in vivo* of estrogens in certain target tissues. In the neonatal mouse, few of the target uterine

epithelial cells are positive for the estrogen receptor at the time of treatment; in contrast, similar treatments of adult animals, when all the cells are estrogen-receptor positive, does not result in DES-induced uterine cancers. In the hamster kidney model, renal tumors are induced by a variety of estrogens, and the tumors that form are estrogen dependent, indicating an important epigenetic mechanism in the genesis and maintenance of this tumor (76). However, not all estrogens are active in inducing these tumors. Tumors are induced by both DES and 17 β -estradiol (E₂), but ethinyl estradiol has only weak carcinogenic activity even though it competes equally well with DES and E₂ for estrogen receptors and has activity similar to carcinogenic estrogens in inducing renal progesterone receptor and serum prolactin levels (80). Similarly, 2-fluorestradiol does not induce renal clear-cell carcinomas in hamsters despite its estrogenic potency (81).

Further evidence for a direct estrogen-induced effect on target cells was provided by studies of neoplastic transformation of Syrian hamster embryo (SHE) cells by DES, E₂, and other estrogens. DES and E₂ induce morphological and neoplastic transformation of SHE cells that is indistinguishable from that induced by other chemical carcinogens such as benzo[*a*]pyrene (78,82). In an attempt to understand DES-induced cell transformation, the ability of DES to induce a variety of genetic changes in SHE cells was examined. Treatment of these cells with DES alone induces cell transformation without causing gene mutations, unscheduled DNA synthesis, sister chromatid exchanges, or structural chromosome aberrations (83,84). Thus, DES can induce cell transformation in the absence of detectable DNA damage. However, under these conditions, DES does induce one type of genetic change, aneuploidy. Diethylstilbestrol binds to microtubules and disrupts tubulin assembly (85-87). Treatment of cells in mitosis with doses as low as 10 nM DES results in aneuploidy induction via nondisjunction (83). Several lines of evidence support the hypothesis that aneuploidy is involved in DES-induced cell transformation (88) and include the following findings: *a*) DES induces significant levels of loss or gain of one or two chromosomes at nontoxic doses; *b*) DES induces aneuploidy and cell transformation with parallel dose-response curves; *c*) aneuploidy induction correlates with the ability to induce cell transformation by DES-related compounds; *d*) cell-cycle specificity of aneuploidy induction and cell transformation by DES indicate that cells in the G₂/M phase are most sensitive; *e*) neoplastic hamster cell lines induced by DES are near-diploid with a nonrandom chromosome change (trisomy 11); and *f*) DES disrupts microtubule organization in cells, providing a biochemical mechanism for induction of chromosome nondisjunction.

In conclusion, it is clear that hormones can affect carcinogenesis by epigenetic mechanisms such as stimulation of cell proliferation of estrogen-dependent target cells and reprogramming of cellular differentiation. In addition, significant evidence exists that certain estrogens can also cause genetic alterations by mechanisms not involving the classical estrogen receptor. These findings indicate that hormonal carcinogenesis is most likely a result of the interplay of both genetic and epigenetic factors.

Table 6. Mechanisms of hormonal carcinogenesis.

Hormonal stimulation of cell proliferation
Heritable reprogramming of cellular differentiation
Induction of genetic changes in target cells either by:
Induction of nondisjunction and aneuploidy via microtubule alterations or
Induction of mutagens following activation to DNA reactive intermediates

Classification of Chemicals by Mechanisms of Action

The somatic mutation theory of carcinogenesis remains the main tenet for explaining the carcinogenic activity of chemicals

Table 7. Possible explanations for nonmutagenic carcinogens and mutagenic noncarcinogens.

Putative nonmutagenic carcinogens
Unusual metabolic activation is required for activity in mutational assays (examples: amitrole and DES).
Mutagenic activity of chemical is limited to chromosomal level, i.e., structural or numerical chromosome changes (examples: benzene, arsenicals, DES, and asbestos).
Chemicals are inhibitors of DNA methylation (examples: 5-azacytidine and ethionine).
Chemicals act as tumor promoters (examples: phenobarbital, 2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin, hormones, and asbestos).
Mutagenic noncarcinogens
Mutagenicity exhibited in test system may not be exhibited <i>in vivo</i> due, for example, to differences in metabolic activation/detoxification or DNA repair.
Mutagenicity of a chemical may be limited to a particular type of genetic change (e.g., aneuploidy); because carcinogenesis requires multiple genetic events of diverse types, a particular chemical mutagen may affect only a single step in the carcinogenesis process.
Mutagenicity per se is not sufficient for carcinogenicity due to lack of proliferation in the target tissue.
Less-than-lifetime carcinogenicity studies may be too short to detect late-appearing tumors.
<i>In vivo</i> rodent models are insensitive to weak mutagens.
DES, diethylstilbestrol.

(89). Some carcinogenic chemicals, however, apparently fail to elicit positive responses in *in vitro* assays for genetic toxicity. Several possible mechanisms or explanations for these putative nonmutagenic carcinogens can be proposed (Table 7).

A problem that exists in most *in vitro* assays is the necessity for exogenous metabolic activation, and the lack of a positive response in a mutation assay may relate to this requirement. Even though considerable advances have been made in this area in the last several years, chemicals with unusual metabolic activation pathways will undoubtedly be discovered. Amitrole and DES are possible examples.

Amitrole, a widely used herbicide, is an animal carcinogen and an inducer of cell transformation (90,91). However, it is inactive as a mutagen in bacterial test systems (90). Thus, it has been suggested that amitrole is a nonmutagenic carcinogen. Over the dose range that induced morphological transformation of Syrian hamster embryo cells in culture, amitrole induced gene mutations at the Na⁺/K⁺ ATPase and hypoxanthine phosphoribosyl transferase loci measured concomitantly in the same cells (91). These findings indicate that amitrole may act via a mutational mechanism and contrast with the negative results observed with bacterial and other mutation assays. Although a variety of mechanisms may account for differences in bacterial versus mammalian cell mutagenesis, the most likely explanation is the necessity for metabolism of amitrole for its activity. SHE cells are able to metabolize a variety of chemical carcinogens to active mutagens and transforming intermediates (92). Kraus et al. (93) have shown that amitrole is metabolized to mutagenic intermediates by peroxidases, including prostaglandin synthetase, which is found in high levels in SHE cells, and lactoperoxidase, a model for thyroid peroxidase. Because the thyroid is the target organ for this carcinogen, these findings suggest that organ-specific metabolism is important in amitrole carcinogenicity.

Many genotoxicity assays measure only the activity of a chemical to induce point mutations or DNA damage. However, chemicals can also induce genetic changes at the chromosomal

level without causing gene mutations or directly damaging DNA (89,94). These chemicals, therefore, would be negative in some genotoxicity assays. Certain exceptions to the correlation between carcinogenesis and mutagenicity based on results in the Ames test (e.g., benzene, arsenic, DES, and asbestos) may relate to the ability of certain chemicals to act specifically as chromosome mutagens (i.e., clastogens and/or aneuploidogens). The data supporting the conclusion that DES and asbestos are chromosome mutagens have already been discussed. Benzene, a known human carcinogen, has been reported as negative in most gene mutation assays, but some positive results have been presented (95-97). However, clear evidence exists that cytogenetic damage is induced by benzene (63), indicating that it is primarily an inducer of chromosome damage, and this is likely its major mechanism of action. Similarly, arsenic and arsenical compounds are known human carcinogens, which are inactive or weak gene mutagens, but very potent clastogens (63). Sodium arsenite and sodium arsenate induce morphological transformation of SHE cells in culture (98). Under these conditions, gene mutations at two genetic loci cannot be detected, but chromosome aberrations and gene amplification (48) are significantly increased, with a similar dose response to that for induction of cell transformation. It is likely that there are other examples of carcinogens that are primarily chromosome mutagens.

Methylation of DNA at the C-5 position of cytosine is important in the regulation of gene expression and is one possible epigenetic mechanism for the heritable change in cancer cells (99). Chemicals such as 5-azacytidine and ethionine may effect DNA methylation through an interaction with the DNA methyltransferase enzyme. It has also been suggested that DNA-alkylating agents may heritably alter DNA methylation patterns (99). This provides an epigenetic mechanism for heritable alterations in expression of genes involved in carcinogenesis.

Other epigenetic mechanisms for carcinogens can be proposed (1). A number of these may involve the tumor-promoting activity of the carcinogenic chemical. It is imperative, however, to remember that genetic and epigenetic mechanisms for a chemical are not mutually exclusive. Many of the chemicals shown to have genetic activity also display epigenetic properties and tumor-promoting activity, which are undoubtedly important in their carcinogenic potential. The thyroid hormone disturbances caused by amitrole (90) and the possible target organ mutagenicity of this chemical are both potential mechanisms that possibly play dual roles in its carcinogenicity. Similarly, DES and other hormonal carcinogens may operate through multiple mechanisms as discussed earlier.

Weisburger and Williams (100) have proposed classification of chemical carcinogens on the basis of mechanism of action into two groups: *genotoxic* and *epigenetic*. Two problems exist with this classification: first, the terminology is problematic, and second, classification implies exclusivity, which is probably rarely the case with a chemical carcinogen. The term genotoxic [which has now been replaced (101) with "DNA reactive" due to the confusion with the original usage of the word by Druckrey] is used to describe specifically carcinogens that undergo chemical reaction with DNA. Carcinogens that are not DNA reactive but display other properties that could underlie an increase in neoplasms (for example, promoting activity), are termed "epigenetic carcinogens" (100). Unfortunately, this definition of epigenetic carcinogen describes the observed action of the

Table 8. Mechanisms of chemically induced mutations in tumors.

Chemical → DNA (adduct) → mutation
Chemical → microtubule (spindle dysfunction) → DNA (aneuploidy) → mutation
Chemical → O ₂ (activated) → DNA → mutation
Chemical → receptor → enzyme → O ₂ (activated) → DNA → mutation
Chemical → receptor → protein (e.g., recombinase) → DNA → mutation
Chemical → receptor → protein → DNA synthesis/cell division (normal) → mutation
Chemical → receptor → protein → DNA synthesis/cell division (abnormal, i.e., mutation rate) → mutation
Chemical → receptor → protein → DNA synthesis/cell division → tumor → mutation

chemical rather than the mechanism of the alteration in cellular phenotype, which is the original context in which this term is used (8). This can create considerable confusion. For example, chemicals that do not react directly with DNA, such as spindle poisons or generators of oxygen radicals, but through indirect mechanisms result in genetic changes such as chromosome rearrangement or aneuploidy, would be called epigenetic carcinogens by Williams and Weisburger's terminology. It is a nonsequitur to call a chemical that induces a genetic change an epigenetic chemical. Likewise, DNA-reactive chemicals (for example, methylating agents) may cause heritable, epigenetic cellular changes by altering DNA methylation (99) or gene expression (102). Should these chemicals be called genotoxic or epigenetic? According to Williams and Weisburger's classification, they are genotoxic, while their mechanism of action may involve an epigenetic change.

Most, if not all, tumors have genetic changes, which may or may not result from mutagenic exposures. It is useful to understand the mechanisms by which carcinogenic chemicals induce genetic changes that arise in chemically induced tumors. Rather than simply dividing the possible mechanisms into two categories, i.e., genotoxic and nongenotoxic, a number of mechanisms of chemically induced mutations in tumors can be envisioned (Table 8). If a chemical induces a cancer and that cancer has genetic changes, it is possible that the chemical directly induced the genetic change, for example, due to a DNA adduct. At another extreme, the chemical may induce the cancer by a nongenetic mechanism, the tumor becomes genetically unstable and mutations arise due to the nature of the tumor rather than the mutation causing the tumor. A number of mechanisms exist between these two extremes. The chemical may induce mutations by indirect mutational mechanisms, e.g., disruption of spindle function or generation of reactive oxygen radicals. These reactive oxygen radicals may arise due to the intrinsic properties of the chemical or due to receptor-mediated production of enzymes increasing rates of oxygen metabolism in cells. Other receptor-mediated changes can indirectly lead to mutations as well, as outlined in Table 8. Any attempt to classify chemical carcinogens by mechanism of mutation induction must consider the complexity and the multitude of possible mechanisms.

Many chemical carcinogens operate via a combination of mechanisms, and even their primary mechanism of action may vary depending on the target cells. For example, some chemicals are complete carcinogens in one tissue, promoters in another, and initiators in another. Classification of chemicals into a single category may be misleading and hinder our comprehensive understanding of the complex problem of chemical carcinogenesis.

The need to understand the mechanism(s) of chemical carcinogens is clearly evident. However, the exercise of classifying chemicals according to mechanism has certain inherent difficul-

ties. A working group of IARC concluded that no classification of chemicals according to mechanisms could be exhaustive or definitive (103). This conclusion is still supported by our current understanding of the molecular basis of multistep carcinogenesis.

One possible advantage of classification of certain chemicals is to distinguish chemicals with different dose-response characteristics, in particular chemicals for which a threshold may exist. This is an area about which too little is currently known to draw any conclusions. It is this author's opinion that chemicals exhibiting a threshold dose response may be identified, but this response will be related to the individual characteristics of a given chemical and not to its characteristic biological activity. Both mutagenic and nonmutagenic chemicals may exhibit thresholds, and likewise chemicals may exert epigenetic effects with a linear dose response. Therefore, no generalizations can be made, and each chemical will require independent analysis.

Role of Cell Proliferation in Carcinogenesis

Cell proliferation can influence carcinogenesis by a number of mechanisms (Table 9). This has led to the hypothesis that cell proliferation per se may be carcinogenic and carcinogens that increase cell proliferation may be operating exclusively by this mechanism. The failure to detect a measurable mutagenic activity associated with nongenotoxic carcinogens indicates that these chemicals may act by alternative mechanisms of action, increasing cell proliferation being one possibility. This hypothesis is supported by the fact that most, if not all, types of cancers may arise spontaneously in at least some species. Normal cell division results in a low level of spontaneous errors during DNA replication, and spontaneous DNA damage can result from cytosine deamination at physiological temperatures, from oxidative damage associated with normal cellular physiology, and from mutagens in food, air, or water (104). Thus, mutations occur "spontaneously" from normal cellular processes. There are risk factors for human cancers (e.g., hormones) that also influence the rate of cell proliferation in target tissue (73). However, mechanisms in addition to cell proliferation should be considered for these risk factors (*vide supra*).

Table 9. Mechanisms by which chemicals affecting cell proliferation might influence carcinogenesis.

Increase fixation and expression of premutagenic DNA lesions
Increase the number of initiated cells occurring spontaneously during cell replication
Increase the number of spontaneous initiated cells by blocking cell death/elimination
Increase the number of initiated cells by perturbing checkpoints in the cell cycle leading to mutagenic events
Increase the rate of neoplastic progression by previous four mechanisms
Promote clonal expansion of initiated cells

Table 10. Evidence against cell proliferation per se being carcinogenic.

Many toxic and/or hyperplastic stimuli are noncarcinogenic.
Cell division occurs frequently in all organisms ^a
For humans:
1 egg → 10 ¹⁴ cells in adult organism
10 ¹³ cells still capable of cell division
10 ⁷ cell divisions/sec occur in adult organism
10 ⁶ cell divisions/sec in intestine
Multiple mutations (3–4?) are required for a normal cell to evolve into a cancer cell.

^aD. Prescott, personal communications.

Before cell proliferation per se can be accepted as the causative mechanism for certain carcinogens, several facts should be considered (Table 10). First, many toxic and/or hyperplastic stimuli are not carcinogenic (105–108). A review of the literature in this field and further studies of noncarcinogenic, toxic agents are needed. Second, cell division occurs frequently in all organisms (Table 10); therefore, it is not clear whether cell division is limiting in the carcinogenic process. This, of course, depends on the target tissue. Furthermore, cell division of initiated or intermediate cells may occur at quite different rates than division of normal cells. Finally, the observation that multiple mutations are involved in the development of many neoplasms may suggest that even a weak mutagenic response, which is below the level of detection of current assays, is sufficient to influence the neoplastic process in a specific target tissue. This is a plausible explanation for certain nongenotoxic carcinogens, some of which may act by indirect mutagenic processes.

Summary

Mutational mechanisms can be proposed for most, if not all, known human carcinogens. Many of these chemicals are electrophilic or metabolically activated to reactive molecules that can alter DNA, causing genetic damage and different types of mutations. Even some previously proposed nongenotoxic human carcinogens (e.g., hormones and asbestos) exhibit mutational activity when assays for chromosomal mutations are used. Because these chemicals are usually inactive in the Salmonella assay and other gene mutation assays, more emphasis has been placed on their nonmutational mechanisms. Clear evidence exists that these carcinogens can alter gene expression and stimulate cell proliferation by epigenetic mechanisms. These properties are undoubtedly important in the carcinogenic activity of these chemicals. Although less well studied, DNA-reactive, genotoxic carcinogens also alter gene expression and increase cell turnover by epigenetic mechanisms.

These findings are consistent with the current understanding of the molecular basis of multistep carcinogenesis. The neoplastic evolution of most common human cancers occurs as the result of multiple mutational events. The molecular basis for these mutations is varied and includes point mutations, deletion mutations, chromosome rearrangements, gene amplification, and chromosome losses and gains. Therefore, different mutational activities of carcinogens can influence the carcinogenic process at different steps. In addition, chemical influences on gene expression and cell proliferation are important in allowing clonal expansion of preneoplastic cells and in disrupting the suppressive effects of surrounding normal cells on preneoplastic cells (109).

The mechanisms of action of human carcinogens, and likely many rodent carcinogens, will include both genetic and epigenetic processes. Carcinogenesis is a multistep, multigenic, multicausal process (8). As such, both epigenetic and genetic factors are probably important. Thus, it should not be surprising that chemicals that are carcinogenic often have the ability to induce both types of changes. These mechanisms are not mutually exclusive; rather, they probably work in conjunction to result in neoplastic progression.

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